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Viral vectors for use in the development of biodefense vaccines

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Abstract

The heightened concerns about bioterrorism and the use of biowarfare agents have prompted substantial increased efforts towards the development of vaccines against a wide range of organisms, toxins, and viruses. An increasing variety of platforms and strategies have been analyzed for their potential as vaccines against these agents. DNA vectors, live-attenuated viruses and bacteria, recombinant proteins combined with adjuvant, and viral- or bacterial-vectored vaccines have been developed as countermeasures against many potential agents of bioterrorism or biowarfare. The use of viruses, for example adenovirus, vaccinia virus, and Venezuelan equine encephalitis virus, as vaccine vectors has enabled researchers to develop effective means for countering the threat of bioterrorism and biowarfare. An overview of the different viral vectors and the threats they counter will be discussed.

Keywords: Vaccine; Viral vectors; Biodefense; Bioterrorism

Abbreviations: Ad, adenovirus; BoNT, botulinum neurotoxin; BWC, Biological and Toxin Weapons Convention; C, capsid; Con, Connaught strain; EBOV, Ebola hemorrhagic fever virus; EBOV(Z), Ebola hemorrhagic fever virus Zaire strain; ELISA, enzyme-linked immunosorbent assay; FDA, Food and Drug Administration; GP, glycoprotein; HA, influenza virus hemagglutinin protein; Hc, carboxy-terminal fragment from the heavy chain of botulinum neurotoxin; HFRS, hemorrhagic fever with renal syndrome; HIV, human immunodeficiency virus; HPS, huntavirus with pulmonary syndrome; HTNV, Hantaan virus; kb, kilobases; kbp, kilobase pairs; KHF, Korean hemorrhagic fever; LSV, Lassa virus; LSGPC or LGP, Lassa virus envelope glycoprotein; LSN or N or LNP, Lassa virus nucleoprotein; MBGV, Marburg virus; mSEB, mutagenized staphylococcal enterotoxin B; NHP, nonhuman primate; NIAID, National Institute of Allergy and Infectious Diseases; NP, nucleoprotein; NYBH, New York Board of Health; OVA, chicken ovalbumin; PA, protective antigen; pfu, plaque forming units; PUUV, Puumala virus; RCN, raccoon poxvirus; RP, replicon particle; RV, rabies virus; RVFV, Rift Valley fever virus; SEB, staphylococcal enterotoxin B; SEOV, Seoul virus; SFV, Semliki Forest virus; SINV, Sindbis virus; TK, thymidine kinase; TRD, Trinidad donkey; UNSCOM, United Nations Special Commission; UTR, untranslated region; VEEV, Venezuelan equine encephalitis virus; VHF, viral hemorrhagic fever; VRP, Venezuelan equine encephalitis virus replicon particle; VSV, vesicular stomatitis virus; VV, vaccinia virus.

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14. ABSTRACT

The heightened concerns about bioterrorism and the use of biowarfare agents have prompted substantial increased efforts towards the development of vaccines against a wide range of organisms, toxins, and viruses. An increasing variety of platforms and strategies have been analyzed for their potential as vaccines against these agents. DNA vectors, live-attenuated viruses and bacteria, recombinant proteins combined with adjuvant, and viral- or bacterial-vectored vaccines have been developed as countermeasures against many potential agents of bioterrorism or biowarfare. The use of viruses, for example adenovirus, vaccinia virus, and Venezuelan equine encephalitis virus, as vaccine-vectors has enabled researchers to develop effective means for countering the threat of bioterrorism and biowarfare. An overview of the different viral vectors and the threats they counter will be discussed.

15. SUBJECT TERMS

bioterrorism, biowarfare, BW, threat, vaccine, recombinant, review, vaccine

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Contents

1. Introduction

The use of biological agents to poison or infect humans and animals has been reported throughout history. Early biological attacks in the 14th century using cadavers who died from plague against the city of Kaffa or soldiers in the 18th century using blankets from a smallpox hospital against Native Americans may have initiated epidemics in the naive target populations [1]. The number of casualties from such acts is difficult to determine due to the complex epidemiological and endemic disease situation. Paralleling advances in microbiology and biotechnology, the production and dissemination of biological agents has become more sophisticated. During World War II, many nations including the United States, the United Kingdom, Japan, Germany, and the Former Soviet Union developed biological weapons for use on the battlefield. The U.S. offensive biological agent research program began in 1942 under the control of a civilian agency, the War Reserve Service, and was locate at Camp Detrick. Anthrax, plague, typhus, cholera, and typhoid were investigated for potential use in biowarfare. Some agents like *Bacillus anthracis* (the etiological agent of anthrax), botulinum neurotoxin (BoNT), and *Francisella tularensis* (the etiological agent of tularemia) were weaponized before the Convention on the Prohibition and Stockpiling of Bacteriological (Biological) and Toxin Weapons and on Their Destruction of 1972, commonly known as the Biological and Toxin Weapons Convention (BWC) [2].

Prior use of biological agents by radical groups has highlighted the need for better surveillance and

control of biological agents [1]. In 1984 the Rajneeshees in The Dalles, Oregon, tainted salad bars with *Salmonella* to try to influence the outcome of a local election. Another radical group in Japan, the Aum Shinrikyo, allegedly conducted research on BoNT, *B. anthracis*, and *Coxiella burnetii*. Their attempts at disseminating *B. anthracis* from the tops of tall building in downtown Tokyo, Japan, were unsuccessful because they failed to anticipate the meteorological conditions. These events, along with other events not mentioned, show that in pursuit of their goals, small groups can obtain, prepare, and disseminate biological agents. A major concern for nations around the world has been state-sponsored programs for the development of biological agents, considered biological warfare agents, for producing mass casualties,. Even though the BWC prohibited offensive research, instances of state-sponsored research continued into the early 1990s.

Between 1985 and 1991, Iraq had weaponized *B. anthracis*, BoNT, and aflotoxin and actively researched *Clostridium perfringins*, rotavirus, echovirus 71, and camelpox virus for use in biological warfare [2,3]. Their arsenal contained approximately 200 bombs and 25 ballistic missiles filled with biological agents which were deployed before the onset of the Persian Gulf War. After the war, the United Nations Special Commission (UNSCOM) questioned Iraqi scientists and inspected dozens of potential manufacturing facilities throughout Iraq. The UNSCOM inspections led to the destruction of Iraq's reported arsenal, yet discrepancies were apparent between amounts produced and amounts destroyed. The current search for weapons of mass destruction, starting with Operation Enduring Freedom and continuing with Operation Iraqi Freedom, 2003, has found no weapons of mass destruction. Even so, other rogue nations and terrorists may have some of acquired Iraq's biological agents and may be attempting to develop or disseminate them.

In 1992, Russia disclosed that they had continued developing offensive biological weapons after the 1972 BWC prohibition and that the facility in Sverdlovsk was conducting offensive biological research and was responsible for the anthrax incident that had happened there in 1979 [1]. They also stated that they would no longer conduct offensive biological research. At its peak during the 1970s and 1980s, the Russian biological program was thought to have employed some 55,000 scientists and technicians at six research laboratories and at five production facilities. A 1995 report estimated that Russia still employed 25,000–30,000 people in their biological defense research programs [4].

During the fall of 2001, letters filled with *B. anthracis* spores (the causative agent of anthrax) were mailed to prominent leaders in the U.S.. The spores contained in the letters resulted in 22 confirmed or suspected anthrax infections [5]. Eleven cases of inhalational anthrax leading to 5 deaths and 11 cases of cutaneous anthrax (7 confirmed, 4 suspected) were reported. Active surveillance of an estimated 10.5 million residents from New Jersey, Washington, DC, Pennsylvania, Maryland, and Virginia did not identify any additional cases of inhalational anthrax. This event strongly suggests that individuals or groups in this country may be actively engaged in developing biological agents for use in terrorism.

Countermeasures against possible wartime biological attacks and against peacetime acts of bioterrorism are currently focused on the development of effective antisera, vaccines, and therapeutic agents. The National Institute of Allergy and Infectious Diseases (NIAID) has classified biological organisms and toxins as category A, B, or C priority pathogens (Table 1). This review will cover the vaccines that have been developed for a majority of the category A and B agents (excluding dengue virus which has been reviewed elsewhere [6]). The prioritization has helped to focus resources on areas that are of the highest concern to the U.S. and to the world. The development of vaccines against the different threat agents can be divided into three areas.

The first area comprises vaccines made from purified "naked" DNA. The naked DNA encodes all the necessary elements for expression of the vaccine gene in mammalian hosts. The DNA is delivered by gene gun or by needle into the recipient. Ease of construction and purification of the DNA vaccines makes them attractive candidates for development, yet, poor antibody responses in the vaccine recipients and concerns over chromosomal integration has limited some of this development [7].

The use of killed viruses or bacteria, or recombinant proteins in the formulation of vaccines covers the

Table 1
National institute of allergy and infectious diseases (NIAID) category A, B, and C priority pathogens

Category A	Category B
Bacillus anthracis (anthrax)	*Burkholderia pseudomallei*
Clostridium botulinum (botulinum neurotoxin)	*Coxiella burnetti* (Q Fever)
Yersinia pestis (plague)	*Brucella* species (brucellosis)
Variola major (smallpox) and other pox viruses	*Burkholderia mallei* (glanders)
Viral hemorrhagic fevers	Ricin toxin (from *Ricinus communis*)
Arenaviruses	Epsilon toxin of *Clostridium perfringens*
LCM, Junin virus, Machupo virus Guanarito virus, Lassa fever	*Staphylococcus* enterotoxin B
Bunyaviruses	Typhus fever (*Rickettsia prowaxekii*)
Hantaviruses	Food and waterborne pathogens
Rift Valley fever	Bacteria
Flaviviruses	Diarrheagenic *E. coli*
Dengue	Pathogenic *Vibrios*
Filoviruses	*Shigella* species
Ebola	*Salmonella*
Marburg	*Listeria monocytogenes*
	Campylobacter jejuni
	Yersinia entercolitica
	Viruses (Caliciviruses)
Category C	Protozoa
Tickborne hemorrhagic fever viruses	*Cryptosporidium parvum*
Crimean–Congo Hemorrhagic fever virus	*Cyclospora cayatanensis*
Tickborne encephalitis viruses	*Giardia lamblia*
Yellow fever	*Entamoeba histolytica*
Multidrug-resistant TB	*Toxoplasma*
Influenza	Microsporidia
Other Rickettsias	Additional viral encephalitides
Rabies	West Nile virus
	LaCrosse
	California encephalitis
	VEE
	EEE
	WEE
	Japanese encephalitis virus
	Kyasanur Forest virus

http://www.niaid.nih.gov/biodefense/bandc_priority.htm.

second area. Recent developments in vaccine adjuvant technology have greatly improved the immunogenicity of proteins used in these vaccines [8]. Past efforts focused on using whole-cell formulations or whole toxin preparations treated with formaldehyde, while today's advancements in biotechnology has allowed a more focused approach through the use of recombinant subunit vaccines [9].

The third area of research involves the use of live-attenuated viruses or bacteria as vaccines candidates or the use of live-attenuated bacteria or viruses as vectors for expressing heterologous vaccine genes in vaccine recipients. Examples of live-attenuated organisms used in biodefense vaccines includes TC-83, a cell culture-derived Venezuelan equine encephalitis virus (VEEV) vaccine [10,11], and Live Vaccine Strain, an attenuated strain of *F. tularensis*, for protection against tularemia [12]. Live-attenuated vaccines have the advantage in that only one inoculation is usually required to stimulate a protective immune response while the potential disadvantages include reactogenicity and concerns over the reversion of the attenuated organism to a virulent state [13]. Bacterial vectors, specifically *Salmonella enterica*, have been used to express vaccine-related genes in animals and to produce biodefense vaccines [14,15]. Viruses have also been used to construct biodefense vaccines and can be separated into two different groups. The first group is composed of replication-competent viruses, e.g., chimeric viruses; and the second group is composed of replication-defective viruses, e.g., replicons. Viruses in each group act as vectors for delivering and expressing vaccine-related genes in animals. This review will focus on the use of replication-competent and -defective viruses as vectors in the development of biodefense vaccines.

2. Poxvirus vectors used in development of biodefense vaccines

2.1. Vaccinia virus-vectored vaccines

Many reports have focused on the development of vaccinia virus (VV) as a vaccine vector for protection against a wide range of pathogens. VV is an orthopoxvirus that belongs to the Poxviridae family, a large family of complex DNA viruses [16]. Their genome consists of a single, linear, double-stranded DNA molecule of 130 to 300 kilobase pairs (kbp) with a hairpin loop at each end. Two subfamilies, Entomopoxvirinae (insect poxviruses) and Chordopoxvirinae, (vertebrate poxviruses) have been described. Two notable genera in Chordopoxvirinae are the *Orthopoxvirus* genus containing the monkey-

pox, camelpox, VV, smallpox, raccoonpox, and cowpox viruses and the *Avipoxvirus* genus containing the canarypox and fowlpox viruses.

The VV-vectored vaccines are generally constructed by employing homologous DNA recombination in VV-infected cells [17]. The foreign gene of interest is inserted into a shuttle vector flanked by poxvirus sequences. Expression of the foreign gene is regulated by insertion of the gene downstream of either an early, intermediate, or late VV promoter. The shuttle vector is then transfected into mammalian cells infected with VV, or co-transfected with VV DNA into helper virus-infected cells. By targeting the thymidine kinase (TK) gene as the site for homologous recombination, recombinant virus can be selected for in TK-deficient cells. Additional methods for preparing recombinant VV include direct ligation of a gene of interest into VV followed by transfection into cells infected with helper virus. In particular to biodefense, there have been several reports that have described the immunogenicity and efficacy of VV-vectored vaccines against Lassa virus (LSV), Ebola hemorrhagic fever virus (EBOV), anthrax, VEEV, and *Brucella abortus* and one report of using raccoon poxvirus as the vector for a plague vaccine. Three other poxviruses, lumpy skin disease virus (*Capripoxvirus* genus) [18], Orf virus (*Parapoxvirus* genus) [19], and fowlpoxvirus (*Avipoxvirus* genus) [20], have been modified to express vaccine-related genes but have not yet been used to construct biodefense vaccines. Of interest is that the lumpy skin disease virus vector was designed as a replication-deficient vector whereas the Orf virus vector and the fowlpoxvirus vectors were both replication-competent. The large variety of poxviruses that might be use to construct vaccine vectors adds to the versatility of using these viruses in the development of biodefense vaccines.

2.1.1. Vaccinia virus-vectored Lassa fever vaccines

NIAID classifies the viral hemorrhagic fever (VHF) viruses as category A pathogens because of their potential for aerosol dissemination and for their ease of weaponization [21,22]. The VHF viruses are highly infectious by aerosol and are easily grown in cultured cells. These properties and the public's perception of the explosive EBOV outbreaks that have occurred in Africa make the viral VHF viruses ideal candidates for use in bioterrorism and biowarfare. The VHF viruses include LSV, Junin, Machupo, Guanarito, Sabia, EBOV, Marburg virus (MBGV), Crimean–Congo hemorrhagic fever, Hantaan, Seoul virus (SEOV), Rift Valley fever virus (RVFV), dengue, yellow fever, Omsk hemorrhagic fever, and Kyasanur Forest disease virus.

LSV, the etiological agent of Lassa fever, is a member of the Arenaviridae family and was first described in West Africa in 1969 [23,24]. Lassa fever is naturally acquired through inhalation of dust contaminated with rodent excreta containing LSV and has a case fatality rate of approximately 15% to 20%. LSV exhibits a pleomorphic morphology containing a bi-segmented RNA genome (small and large segments) with an ambisense organization. The viral particles consist of a host cell-derived lipid envelope containing the viral glycoproteins (GP) GP1, or G1, and GP2, or G2 surrounding a helical nucleocapsid.

VV-vectored vaccines against LSV have been constructed which express the LSV precursor envelope GP (LSGPC) gene in the New York Board of Health (NYBH) strain of VV (also referred to as the WYETH strain) [25]. Guinea pigs inoculated with V-LSGPC did not produce antibodies to LSV before challenge, but were protected from a lethal LSV challenge (11 of 11 animals survived). Upon challenge, the animals developed low-grade fevers and became viremic but the viremia was 10-fold lower than that observed in the control animals. Guinea pigs vaccinated with a recombinant VV (Lister strain) expressing Lassa virus NP (LSN) were completely protected from disease following a LSV challenge [26]. No viremia was detected in the six vaccinated animals during the 28-day study.

Additional VV (NYBH strain)-vectored LSV vaccine studies in guinea pigs produced similar results. Guinea pigs inoculated with V-LSGPC, V-LSN, or both at different sites, were 79%, 94%, or 58% protected, respectively, from LSV challenge as compared to the negative control animals (39% survived challenge) [27]. Of the vaccinated guinea pigs, 52 of the 60 animals developed fevers and 57 of the 60 animals were viremic after challenge. In a different study, VV (NYBH strain)-vectored vaccines expressing both LSN and LSGPC (V-LSGN-II) were used to vaccinate mice [28]. Mice inoculated with V-LSGN-II produced antibody titers against LSV and

against VV. An increase in antibody to both LSV and VV was noted after a second inoculation. The antibody titer to LSV elicited by inoculation of mice with the double gene vaccine was 2-fold higher than that achieved after inoculation with either of the single gene vaccines. Since mice do not succumb to Lassa fever, they were not challenged.

To better understand the vaccine requirements necessary to protect humans from Lassa fever, non-human primates (NHPs) were vaccinated with the V-LSGPC vaccine (NYBH strain of VV) as described above [29]. Two of the four monkeys vaccinated with V-LSGPC produced antibodies specific for LSV. After challenge, the four monkeys developed a febrile illness with low viremia and survived challenge. In a subsequent study, groups of monkeys were vaccinated with LSN, LSG1, LSG2, LSG1, and LSG2 at different sites, LSGPC, LSGPC, and LSN at different sites, or with VV expressing both LSGPC and LSN (V-LSG/N) [30]. Vaccines containing both LSG1 and LSG2, with or without LSN, protected the animals from death, whereas LSN, G1, or G2 alone failed to protect the animals from death. Of the 18 surviving animals, 16 developed low-level viremia and two did not have detectable viremia.

The studies described here provide indirect evidence that a VV-vectored LSV vaccine may protect humans from severe Lassa fever and death but may not completely prevent the associated febrile illness. Comparison of the data obtained from the mouse, guinea pig, and NHP studies shows that the VV-vectored LSV vaccines provide protection from death but not from disease. Vaccination of guinea pigs with LSN provided the best protection from death but that same level of protection was not observed in the vaccinated NHP. Vaccination with G1 and G2 was required to protect the NHP from death but such vaccination did not prevent them from developing viremia and illness. The difference in vaccination requirements may involve the difference between cell-mediated protection (that elicited by LSN vaccination), which may have provided some protection to the guinea pigs, as compared to antibody-mediated protection (that elicited by glycoprotein vaccination), which may have provided some protection to the NHP. Additional studies focused on increasing the immunogenicity of the VV-vectored vaccines and the use of different strains of VV may produce a vaccine that better protects against this disease.

2.1.2. Vaccinia virus-vectored Ebola hemorrhagic fever vaccines

First recognized as a serious disease in humans in Africa in 1976, EBOV has caused numerous outbreaks since and remains a serious public health threat throughout Central and West Africa [31]. EBOV contains a non-segmented, negative-sense, single-stranded RNA genome and exhibits a filamentous branched or circular morphology. The approximately 19 kilobase (kb) viral genome is surrounded by NP and a host cell-derived lipid envelope containing the viral glycoprotein, GP. Vaccines against category A pathogens that cause hemorrhagic fevers like EBOV have been constructed and evaluated for immunogenicity and protective efficacy in animals. A VV vector expressing the GP gene from EBOV, a member of the Filoviridae family, has been evaluated in guinea pigs and NHPs. Strain 13 guinea pigs inoculated with vGP produced detectable antibodies and were partially protected (three of five animals survived) from 1000 plaque-forming units (PFU) of EBOV [32,33]. No viremia was detected 7 days after challenge in the survivors. Inoculating guinea pigs with VV expressing any of the other viral genes (either NP, VP35, VP40, or soluble GP) did not protect any of the animals from challenge. When the same recombinant vGP was used in NHPs, the results were far different from those observed in the guinea pigs [34]. The three NHPs inoculated with three doses of VACV-GP developed viremia on day 3 and all died on day 6 or 7 after challenge with 1000 PFU of EBOV. Inserting additional vaccine genes into VV, followed by evaluation of immunogenicity and efficacy in NHPs, may result in a candidate vaccine for human use.

2.1.3. Vaccinia virus-vectored Venezuelan equine encephalitis vaccines

VEEV is endemic in many tropical countries in Central and South American and is transmitted by the bite of infected mosquitoes [35]. VEEV is an *Alphavirus* in the Togaviridae family. VEEV exhibits a spherical morphology and consists of a capped, positive-sense single-stranded polyadenylated genome (resembles an mRNA molecule of about 12 kb) surrounded by capsid protein and enclosed in a host

cell-derived lipid envelope containing the viral GP E1 and E2 [36]. Recent large outbreaks involving tens of thousands of individuals and animals, especially horses, have been reported [37]. The current human-use vaccine against VEEV, TC-83, was produced by serial passage of the virulent Trinidad donkey (TRD) strain of VEEV in cultured cells [10]. Reactogenicity of up to 40% in recipients of TC-83 warrants the development of a new VEEV vaccine [11,38]. A live-attenuated VEEV vaccine, V3526, was developed to replace TC-83 and is currently in scale-up production for eventual use in human clinical trials [39]. VEEV was weaponized and stockpiled by the U.S. before the 1972 BWC and is currently classified by NIAID as a category B pathogen [1]. Even though VEEV is classified as a category B pathogen, past development of VEEV by the U.S. and Soviet bioweapons programs as a potential incapacitating agent supports the need for an improved, non-reactogenic vaccine for use in at-risk personnel and as a biowarfare deterrent.

A potential recombinant VV (NYBH Wyeth Laboratories VACC strain) vaccine expressing the capsid protein and the E1 and E2 GP from TC-83 (VACC/TC-83, also called VACC/TC-5A) was evaluated in mice [40]. The VACC/TC-5A vaccine protected 100% of the A/J and C3H mice and 92% of the Swiss NIH mice from a virulent VEEV TRD challenge. Also, VACC/TC-5A protected the mice against challenge with the 1C and 1D variants and subtype 2 VEEV. Eliciting protection against an aerosol exposure to a biological agent is a crucial consideration that determines its usefulness as a biodefense vaccine. In the case of VACC/TC-5A, the vaccine failed to protect the mice from an intranasal challenge with virulent VEEV and thus would probably not be efficacious at protecting individuals from an aerosol exposure. Similar results were noted using a modified VV WR strain (WR103) expressing all of the VEEV structural genes (E3-E2-6K-E1) [41,42]. The VV was modified by inserting a synthetic promoter upstream of the VEEV structural protein genes resulting in a 3.59-fold increase in expressed protein. However, the increased protein expression did not result in an increase in the immunogenicity of the vaccine. The vaccine protected 100% of the mice against a subcutaneous challenge but failed to protect more than 20% of the mice from an aerosol challenge. Substantial increases in immunogenicity and efficacy are needed to warrant additional evaluation of this vaccine formulation.

2.1.4. Vaccinia virus-vectored hantavirus vaccines

Hantaviruses are members of the Bunyaviridae family and are transmitted to humans through contact with aerosolized rodent urine and excreta containing the virus [43–45]. Hemorrhagic fever with renal syndrome (HFRS), hantavirus pulmonary syndrome (HPS), and Korean hemorrhagic fever (KHF) are just a few examples of the diseases caused by hantaviruses. The hantavirus genome is composed of three negative-sense, single-stranded RNA molecules (small, medium, and large segments) surrounded by NP and a host cell-derived lipid envelope containing the viral GP G1 and G2. Hantaviruses are found throughout the world and no U.S. Food and Drug Administration (FDA)-licensed vaccine exists for use in the U.S. but a vaccine for KHF (Hantavax) is in use in the Republic of Korea [46]. Historically, hantaviruses were not developed as biological weapons, but the stability and ease of infection by aerosolized virus has prompted NIAID to classify these viruses as category A pathogens. Recombinant VV expressing the NP and GP genes from Hantaan virus (HTNV) has been evaluated as a vaccine against different hantaviruses in animals. Homologous recombination was use to place the HTNV NP gene downstream of the VV 7.5K promoter and the HTNV GP genes downstream of the 11K promoter in the Connaught (Con) strain of VV (termed VACV vaccine) [47,48]. Syrian golden hamsters inoculated with the recombinant VV were protected from challenge with homologous HTNV and from a heterologous hantavirus, SEOV, but were not protected from another heterologous hantavirus, Puumala virus (PUUV) [49]. Protection was determined by the presence or absence of reverse transcription-polymerase chain reaction assay-detectable virus in the blood of the animals 28 days after challenge. A second group working with recombinant VV also reported similar results with hantavirus strain R22 [50]. The hantavirus R22 strain was isolated from a rat in China and the NP or the GP genes were introduced into VV Wyeth strain to generate RNV or RMV9, respectively. Based upon a lung biopsy assay, RMV9 completely protected Mongolian gerbils from either a homologous R22 challenge or from

a heterologous HTNV challenge whereas RNV was only partially protective.

Data collected during a Phase I dose-escalation study in 16 human volunteers showed that a recombinant VV (Con strain) expressing the G1, G2, and NP proteins (the VACV vaccine described above) could stimulated neutralizing antibody responses to HTNV [51]. The volunteers were divided into four groups, group 1 contained individuals with a history of receiving VV vaccine, and the three remaining groups, 2 through 4, were composed of VV-naïve individuals. Individuals from group 1 had a measurable VV neutralization antibody titer before the initial VACV inoculation and failed to produce a neutralizing antibody responses to HTNV after inoculation with 3.4×10^5 PFU of VACV. Low doses of VACV (group 2 received 3.4×10^5 and group 3 received 3.4×10^6 PFU) only stimulated neutralizing antibody responses to HTNV in 1 of 4 individuals per dose group, while a higher dose of VACV (group 4 received 3.4×10^7 PFU) stimulated neutralizing antibody responses to HTNV in 3 of 4 individuals. Booster inoculations with 3.4×10^7 PFU of VACV administered to the same groups approximately 1 year after their initial vaccinations failed to stimulate anti-HTNV antibody responses in the VV-preimmune individuals but did stimulate neutralizing antibody responses in 1 of 1 individual in group 2, 4 of 4 individuals in group 3, and 2 of 3 individuals in group 4. Of interest was the observation that the individuals in group 3 exhibited a delayed response to the booster vaccination, more indicative of a primary response than an anamnestic response, while the individuals in group 4 exhibited a rapid anamnestic response, but the response was 2-fold to 4-fold lower than that observed after their initial inoculation. Anti-VV immune responses were measured in 10 of the 12 VV-naïve individuals after their initial VACV inoculation and in 4 of 4 VV-preimmune individuals and may have contributed to the varied responses measured in the individuals after their initial and booster inoculations.

Based on the Phase I clinical trial results, a Phase 2 trial was conducted and showed similar results [51]. Of the VV-naïve individuals inoculated with one or two doses of VACV, only 4 of 9 (44%) or 31 of 43 (72%) of the individuals produced neutralizing antibody responses to HTNV, respectively. The second dose of VACV was administered 42 days after the first inoculation. Fewer individuals that had a history of VV immunization responded to the VACV vaccine. Only 1 of 13 (8%) or 12 of 47 (26%) of the VV-preimmune individuals inoculated with one or two doses of VACV produced neutralizing antibody responses to HTNV, respectively. Of those individuals that did respond to VACV, the highest neutralizing antibody titers to HTNV were among the VV-naïve individuals that received two inoculations of VACV. These clinical trials show that VV-vectored vaccines can stimulate immune responses in VV-naïve humans but that pre-existing immunity to VV (see Section 6) interferes with the immunogenicity of the vaccine. The development of VV vectors that elicit less anti-vector immune responses would enhance the immunogenicity of VV-vectored vaccines and would make them more clinically relevant.

2.1.5. Vaccinia virus-vectored anthrax vaccines

Anthrax is a disease normally associated with herbivores and occurs worldwide, including many states in the U.S., with human infection usually resulting from handling contaminated meat and animal products [52]. Inhalation, gastrointestinal, and cutaneous anthrax can result from inhaling spores during the processing of animal products, ingesting spores in contaminated meat, or by exposing an open wound to spores, respectively. Untreated inhalation or gastrointestinal anthrax has a case fatality rate approaching 100% while untreated cutaneous anthrax has a case fatality rate of up to 25%. Early and aggressive antibiotic treatment can prevent disease-associated morbidity and mortality. *B. anthracis*, the etiological agent of anthrax, contains two virulence-associated plasmids. Plasmid pXO1 encodes the capsular antigen responsible for inhibiting macrophage function and the second plasmid, pXO2, encodes the anthrax toxin genes: edema toxin (composed of edema factor and protective antigen) and lethal toxin (composed of lethal factor and protective antigen). The use of *B. anthracis* as a biological weapon dates back to World War I (WW I) when the Germans attempted to infect horses and mules intended for export to Allied Forces [1]. During WW II, the Japanese experimented with numerous pathogens including *B.*

anthracis at their research facility located at Unit 731, near the city of Pingfan. The U.S. also began research into using *B. anthracis* as a biological weapon during WW II and filled 5000 bombs with *B. anthracis* spores produced at a pilot plant at Camp Detrick. The U.S. continued offensive research during the 1950s and 1960s until President Nixon terminated the program in 1969. Iraq admitted to United Nations' inspection teams of having performed research into weaponizing *B. anthracis* for offensive use [1,3] and Russian President Boris Yeltsin's acknowledgement of the true nature of the accidental release of *B. anthracis* spores from the military facility in Sverdlovsk [53] forced the U.S. military and other research groups into devoting considerable resources for developing countermeasures against anthrax. The biowarfare and bioterrorism threat associated with *B. anthracis* has been emphasized by the recent use of *B. anthracis* spores against members of the U.S. Congress [5,52]. The threat associated with the misuse of *B. anthracis* prompted NIAID to classify this organism as a category A pathogen. The current vaccine licensed for human use requires a six-dose primary series and yearly boosters and causes reactogenicity in up to 30% of vaccine recipients [52,54]. A less reactogenic vaccine requiring fewer inoculations and boosters would be more beneficial and easier to administer to at-risk personnel.

One approach at developing new anthrax vaccines is through the use of viral vectors. A recombinant VV-vectored anthrax vaccine was constructed by cloning the protective antigen (PA) gene from *B. anthracis* into the Con and WR strains of VV [55]. WR-PA consistently elicited higher antibody titers than Con-PA in mice with 4.1-fold and 1.4-fold higher titers in ICR and C57BL/6 mice, respectively. Vaccinating mice or guinea pigs with WR-PA protected 60% and 50% of the animals, respectively, from a *B. anthracis* Ames strain spore challenge [56]. The Con-PA vaccine failed to protect any of the animals from a spore challenge. Because the WR-PA vaccine was immunogenic in guinea pigs and mice, additional experiments are needed to determine if this vaccine would stimulate protected immune responses in rabbits, the currently accepted model, in addition to NHP, for determining the efficacy of anthrax vaccines [57].

2.1.6. Vaccinia virus-vectored brucellosis vaccines

Brucellosis, also known as undulent fever and Mediterranean gastric remittent fever, is a disease caused by one of seven different intracellular bacterial species of Brucellae and is one of the world's most important veterinary diseases [54,58]. Four of the seven species are pathogenic in humans and result is a nonspecific febrile illness with a mortality rate of about 2–5% for untreated cases. The four pathogenic species are *B. suis*, *B. melitensis*, *B. abortus*, and *B. canis*, the two nonpathogenic species are *B. ovis* and *B. neotomae*, and the last strain, *B. maris*, has unknown pathogenicity for humans. All seven stains are classified as category B pathogens by NIAID. The U.S. began developing *B. suis* as a biological weapon during World War II and field-tested bombs filled with stabilized bacteria during 1944–1945 [59]. Brucellae were ideally suited for weapons development because the estimated infectious dose is between 100 and 1000 organisms [54]. Further development of Brucellae as a biological weapon by the U.S. also ended in 1969.

Current vaccine research involving Brucellae has focused on using recombinant VV expressing the 18-kDa outer membrane protein of *B. abortus* (v18-1 virus). Mice vaccinated with v18-1 produced antibodies specific for the 18-kDa protein but were not protected from a virulent *B. abortus* challenge. Previous studies identified several proteins to which infected or vaccinated animals develop immune responses, but those responses have not been correlated with protective immunity [60–62]. Evaluating other Brucellae proteins may eventually define those proteins necessary for eliciting protective immunity and may lead to an efficacious vaccine.

2.2. Raccoon poxvirus-vectored plague vaccines

Yersinia pestis, the causative agent of plague and a NIAID category A pathogen, is a rod-shaped, non-sporulating, gram-negative, facultative anaerobic bacterium. The use of *Y. pestis* in warfare dates back to the 14th century when the attacking Tartars tried to initiate a plague epidemic in the city of Kaffa by catapulting plague-infected cadavers into the city [1]. Research conducted by Japan during WW II at Unit 731 focused on developing *Y. pestis* as a biological weapon. Millions of *Y. pestis*-infected fleas were

released from aircraft over Chinese cities in attempts to initiate plague epidemics. The Former Soviet Union dedicated thousands of scientists and committed more than 10 research institutes towards the development of plague as a weapon [63]. The U.S. also researched methods for weaponizing *Y. pestis* during the 1950s and 1960s and discontinued offensive research in 1969. *Y. pestis* is transmitted by the bite of an infected flea and, if left untreated, has a mortality rate of approximately 60% and can develop into the septicemic or pneumonic form of plague. The high mortality rate associated with pneumonic plague, approximately 100% for untreated cases, the highly infectious nature of *Y. pestis* aerosols, the lack of a licensed vaccine for human use, and past development of plague as a weapon has prompted the development of candidate vaccines that can protect against *Y. pestis*.

A recombinant raccoon poxvirus (RCN) expressing the F1 capsule antigen from *Y. pestis* was constructed by homologous recombination of wild-type RCN with a shuttle vector encoding the F1 gene, RCN-IRES-YpF1 [64]. Two additional recombinant viruses were prepared which encoded the tissue plasminogen (tPA) secretory signal, RCN-IRES-tPA-YpF1, or the tPA secretory signal and a canine herpes virus GP G membrane anchor signal, RCN-IRES-tPA-YpF1-gG, in-frame with the F1 gene. Mice inoculated with RCN-IRES-tPA-YpF1 produced high levels of anti-F1 antibodies and were completely protected from a 28 LD_{50} subcutaneous challenge of *Y. pestis* (CO92 strain) compared to 60% protection in mice inoculated with RCN-IRES-tPA-YpF1 and 20% protection in RCN-IRES-tPA-YpF1-gG vaccinated mice. In regards to the use of this vaccine for protection against possible bioterrorism or biowarfare events, further evaluation in an aerosol challenge model would validate this vaccine for advanced development and possible use in humans. The greatest threat from plague arises from the highly infectious nature of aerosols containing *Y. pestis*. The cough from plague patients and the intentional generation of aerosols by terrorists or rogue nations can be extremely infectious and could result in many primary and secondary plague cases. The use of an *Alphavirus* replicon vector (VEEV-replicon, discussed in the next section) expressing a fusion protein between the F1 and V antigen of *Y. pestis* protected 80% of the vaccinated mice against either an aerosolized or parental *Y. pestis* (CO92 strain) challenge (Lee, unpublished data). Taken together, viral vectors expressing *Y. pestis* proteins can stimulate protective immune responses but additional experiments are necessary to develop a vaccine that completely protects against plague.

3. Alphaviruses as biodefense vaccine vectors

Alphaviruses, members of the Togaviridae family, have been successfully used for constructing biodefense vaccines. The viruses contain a 42S single-stranded positive-sense RNA genome encoding four non-structural proteins (providing the replicase and transcriptase function) and three structural proteins (capsid (C), E1, and E2) [65]. The nonstructural proteins are translated directly from the 42S genomic RNA. The structural proteins are translated from a subgenomic, 26S RNA that is transcribed from the full-length negative strand. The 26S promoter drives transcription of the 26S RNA to levels 10 times that of the 42S genomic RNA leading to amplified expression of genes under the control of the 26S promoter. The RNA genome is about 11.5 kb, capped on the 5′ end with N^7-methyl-gaunosine, and polyadenylated on the 3′ end. Replication and gene expression all occur in the cytoplasm of infected cells, thus avoiding the possibility of RNA splicing and integration into the host genome. Construction of alphavirus-vectored vaccines includes converting the viruses into self-replicating RNA replicons removing the structural protein genes and inserting a foreign gene of interest. Co-transfection of cells in vitro with a recombinant replicon and helper RNA molecules, encoding the structural proteins, produces propagation-deficient replicon particles (RPs). Foreign genes of up to approximately 5 kb have been inserted into the replicon and assembled into RP whereas genes larger than 5kb fail to assemble into RP properly. When administered to an animal, the RPs infect host cells but do not produce progeny viral particles. Three replicons, Sindbis virus (SINV)-replicon, VEEV-replicon, and Semliki Forest virus (SFV)-replicon, have been modified to express foreign proteins important in the development of biodefense vaccines. The wide host range and the diverse cells types infected by alphaviruses, coupled with the construction of replicon vectors capable of expressing high

levels of foreign proteins, makes alphavirus replicon vectors ideal candidates for use as vaccine vectors [65,66].

Construction of chimeric alphaviruses containing genes from other viruses has also been useful in vaccine development. These chimeric viruses differ from replicon vectors in that the chimeric viruses are replication-competent viruses whereas the replicon vectors are not replication-competent because they do not contain structural protein genes. Use of replication-competent chimeric viruses may reduce the number of inoculations necessary to elicit protective immunity to a single inoculation.

3.1. Sindbis virus-vectored vaccines

SINV is one of the least pathogenic alphaviruses for humans and belongs to the Old World *Alphavirus* group, whereas VEEV belongs to the New World *Alphavirus* group [65]. SINV is found throughout Europe, Asia, Africa, and Australia as a bird-associated virus transmitted by mosquitoes. The SINV-replicon was assembled into virus-like particles (VLPs) for inoculation into animals by co-transfecting SINV-replicon RNA and one helper RNA that encoded all of the structural genes into cultured cells [67,68]. High levels of foreign gene expression in cultured cells infected with VLPs and the stimulation of protective immunity in animals make the SINV-replicon a promising vaccine vector [69,70].

3.1.1. Sindbis virus-vectored Rift Valley fever vaccines

Rift Valley fever virus (RVFV) is a *Phlebovirus* and a member of the Bunyaviradae family. NIAID has classified RVFV as a category A pathogen because of its highly infectious nature. The genomic organization and viral structure are similar to those described above for hantaviruses. Originally isolated from sub-Saharan Africa in 1930, the mosquito-vectored zoonotic disease can infect humans and presents as a febrile illness similar to influenza infections with a case fatality rate of about 0.5% [43]. RVFV also infect animals such as sheep, goats, cattle, and buffalo which can lead to spontaneous abortions in pregnant animals and has an animal fatality rate of 5–60%. Infected animals can develop high viral titers, sufficient to infect mosquitoes that feed on them, which can then lead to establishment of the virus in the environment with subsequent human infections [54]. Therefore, besides being a biowarfare and bioterrorist threat, this pathogen may also have enormous impact on the agricultural industry of a nation.

A RVFV vaccine was constructed by inserting the 11-amino-acid 4D4 RVFV neutralizing epitope into either of two permissive sites in the E2 GP or in the secreted E3 glycoprotein genes of SINV [71]. Insertion of the 4D4 epitope did not significantly affect the growth properties of the chimeric compared to the parental virus. Mice inoculated with two doses of RVFV-SINV chimeric virus were 50% protected from a lethal RVFV challenge. This vaccine illustrates the use of a replication-competent virus as a vaccine vector. Inserting larger or additional epitope genes into SINV might increase the efficacy of the SINV-vectored RVFV vaccine.

3.1.2. Sindbis virus-vectored Venezuelan equine encephalitis virus vaccines

A second application of SINV has been in the development of a vaccine against VEEV [72]. The potential of VEEV as a biological warfare agent is discussed above. The VEEV vaccine was constructed by cloning the VEEV GP genes into the SINV-replicon. The *cis*-acting RNA elements of the recombinant genome including the 3′-untranslated region (UTR) and the nonstructural proteins were from SINV while the subgenomic UTR and the structural genes were from TC-83, the vaccine strain of VEEV. The resultant chimeric virus, SIN-83, was replication-competent, highly attenuated, and immunogenic in mice. Mice inoculated with one dose of SIN-83 were completely protected from a VEEV strain 1C or 1D challenge. Also, SIN-83 was non-pathogenic for suckling mice whereas TC-83 caused high mortality when given intracerebrally. Typically, replicon-vectored vaccines do not produce progeny virus after infecting host cells. In this case, the heterologous genes were from a virus of the same genus and the heterologous genes performed the same function as those that were replaced, thus allowing for viral replication and the production of progeny virus.

3.1.3. Sindbis virus-vectored hantavirus vaccines

SINV-replicon expressing either the NP gene (pSINrep5-S) or the GP genes (pSINrep5-M) from SEOV, a hantavirus that causes HFRS, have been

evaluated in Syrian hamster [73]. Hamsters vaccinated with either of the pSINrep5 vaccines produced specific antibody responses to the respective replicon-expressed proteins, except for one animal in each group that did not seroconvert. The postchallenge antibody responses to hantavirus genes not present in the vaccine were quantified as an indirect measure of infection in the vaccinated animals. The authors concluded that the replicon vaccines did not induce sterile immunity and thus did not prevent SEOV infection in the challenged animals. Measuring sterile immunity may or may not have any bearing on the ability of a vaccine to protect against disease. Because no animal model of disease is known for hantaviruses (except HPS caused by Andes virus [74]), determining a vaccine's ability to prevent hantavirus-related diseases in humans is problematic. Measuring viremia in the blood or tissue of challenged animals may help in determining the efficacy of a vaccine but may not convey the true efficacy of that vaccine in humans. Continued testing of animal models may help solve the problem.

3.2. Venezuelan equine encephalitis virus (VEEV)-vectored vaccines

Vaccines against a variety of bacterial, viral, and toxin threat agents have been formulated using VEEV-replicon vectors. VEEV-replicons used in the studies described here were derived from an attenuated VEEV and were assembled into propagation-deficient VEEV-replicon particles (VRPs) using a bipartite helper system [75]. The bipartite helper system is composed of two RNAs, one encoding the C gene and the other encoding the GP genes (E3-E2-6K-E1). The GP genes also contain attenuating mutations that provided an additional level of safety in the unlikely event that multiple RNA recombination events could regenerate replication-competent virus. Packaging systems that use only one helper RNA encoding all of the structural protein genes usually generate replication-competent virus. The enhanced immunogenicity of the VEEV-replicon-vectored vaccines has been attributed to the ability of VEEV to infect dendritic cells as compared to other alphaviruses that do not target dendritic cells [76]. The recent construction of a chimeric virus and a chimeric replicon derived from VEEV and SINV has combined the desirable properties from each virus into a potential delivery system for use in vaccine development [72,77]. The VEEV–SINV chimeric virus is replication-competent, whereas the VEEV–SINV chimeric replicon is replication-defective. Both are highly attenuated and are not pathogenic for mice and the VEEV–SINV chimeric virus and one version of the VEEV–SINV chimeric replicon are expected to infect dendritic cells. The use of a replication-defective VEEV–SINV replicon vector may increase the immunogenicity and safety of vaccines vectored by this replicon but additional experiments are necessary to define the usefulness of chimeric replicon vectors. Even though replication-competent VEEV is classified as a category B pathogen and was weaponized in the past, the replication-defective VEEV-replicon and the VEEV–SINV chimeric replicon vaccine vector are an excellent example of how previous research can be used in the development of beneficial vaccines.

3.2.1. VEEV-vectored Ebola hemorrhagic fever and Marburg virus vaccines

EBOV vaccines vectored by VEEV were constructed by inserting EBOV structural genes VP24, VP30, VP35, VP40, GP, or NP into the VEEV-replicon. Using a mouse-adapted EBOV for challenge, Wilson et al. found that high levels of protection could be induced in mice inoculated with any of the VP replicons, but that protection was dependent on the mouse strain used [78]. Even though the mice survived challenge, viremia was detected in all the animals evaluated on days 4 and 5 after challenge. To determine the role of cytotoxic T lymphocytes (CTLs) in protecting the mice from EBOV infection, VEEV-replicons expressing EBOV NP were used to inoculate mice [79]. The mice produced CTLs specific for EBOV NP and those CTLs exhibited a good effector cell to target cell ratio (0.8:1) for specific lysis of targeted cells in the CTL assay. Adoptive transfer of unfractionated T-cells from mice vaccinated with VEEV-replicons expressing NP into naïve mice protected those mice against a lethal mouse-adapted EBOV challenge. Passive transfer of antibodies from vaccinated mice to naïve mice did not protect them from challenge and did not extend their time to death. The results obtained from these studies conducted in mice point to the role of CTLs in

providing protection against EBOV infections and that the presence of antibodies in the animals is not a marker of vaccine efficacy.

Strain 2 guinea pigs inoculated with replicons expressing either EBOV GP or NP produced antibodies against EBOV but were either partially protected or not protected from a guinea pig-adapted EBOV challenge, respectively [80,81]. The guinea pigs that survived challenge were found to be viremic on day 7 after challenge. In a similar study, strain 13 guinea pigs inoculated with VEEV-replicons expressing GP produced about the same level of antibodies as the strain 2 guinea pigs but were completely protected from a guinea pig-adapted EBOV challenge [80,82]. No viremia was detected in these animals 7 days after challenge. This study was part of a larger study that is described in the next section covering VEEV-replicon vaccines against LSV. The results obtained from these guinea pig studies are consistent with the above-mentioned study using VV-vectored GP and NP in guinea pigs. Inoculating NHPs with VEEV-replicons expressing EBOV GP, NP, or a mixture of both failed to stimulate neutralizing antibody titers and failed to protect any of the animals from challenge [34].

Using different animal models and different strains of EBOV has not clearly identified one model as the animal model of choice for evaluating EBOV vaccines. Immune correlates of protection (i.e. CTLs) have been identified for EBOV infection in mice, but are not correlated to protection in guinea pigs or NHP. Adapting EBOV for use in mice or guinea pigs may not represent the best model for studying EBOV immunology and infection since changes in the virus may change the tissue tropism of the virus and how the disease manifests itself in that animal. It is not understood if a vaccine that can stimulate CTLs in mice or antibody responses in guinea pigs is the appropriate vaccine for use in humans, as NHPs inoculated with the same vaccine were not protected from an EBOV challenge. Vaccines composed of several EBOV proteins or vaccinations involving different prime-boost strategies using DNA, adenovirus (Ad)-vectored (discussed in Section 4), or VV-vectored genes may provide insight into what is necessary to elicit a protective immune response against EBOV. Once a protective immune response has been observed in NHP, then correlates of immunity may be determined and a human use vaccine created.

Marburg virus (MBGV), like EBOV, is a *Filovirus* belonging to the Filoviridae family. This virus is also classified as a category A pathogen and has not been reported to have been weaponized but does have the potential for aerosol dissemination and weaponization by terrorists. Originally recognized in 1967, MBGV has caused few natural outbreaks and remains as a sporadic disease in southeast Africa [31,83]. A vaccine-development strategy similar to the one used for developing EBOV vaccines was used to construct vaccines against MBGV. The structural genes from MBGV, namely GP, NP, VP24, VP30, VP35, and VP40, were cloned into a VEEV-replicon and used to vaccinate animals [84]. Strain 13 guinea pigs inoculated with replicons expressing MBGV GP or NP were completely protected from a lethal MBGV challenge whereas guinea pigs inoculated with replicons expressing VP24, VP30, VP35, or VP40 were not protected. No viremia was detected in the GP- and NP-vaccinated animals when measured 7 days after challenge. NHPs vaccinated with the same replicons expressing GP were completely protected from disease when challenged with MBGV. The replicons expressing NP failed to protect the animals from disease but did protect two of the three animals from death. These results are in marked contrast to those reported for EBOV. A vaccine composed of a VEEV-replicon expressing MBGV GP elicited protection in NHPs whereas VEEV-replicon expressing EBOV GP failed to protect the animals from challenge. Additional studies designed at defining the similarities and differences between MBGV GP and EBOV GP may help define the elements necessary to produce a protective EBOV vaccine.

3.2.2. VEEV-vectored Lassa fever and Ebola hemorrhagic fever vaccines

The first VEEV-replicon-vectored vaccine against LSV used the NP (also referred to as N, also referred to as LNP in the next paragraph, also referred to as LSN in the VV section) gene and a bipartite packaging system to evaluate the safety and immunogenicity of VEEV-replicon-vectored vaccines [75]. Mice inoculated with VEEV-replicon expressing either N or influenza virus hemagglutinin (HA) produced high

levels of antibodies specific to each protein. A series of sequential vaccinations (vaccination were 28 days apart) also showed that the VEEV-replicon vector could be administered multiple times without anti-vector immune responses compromising the effectiveness of the vector. Because they do not develop LSV disease, mice sequentially vaccinated with VEEV-replicons expressing N and then with HA were challenged with influenza virus. None of the mice developed disease from the challenge.

Pushko et al. then used the VEE-replicons to evaluate the efficacy of replicons expressing the LSV GP (LGP) and the EBOV GP (EGP) in guinea pigs [82]. Guinea pigs were vaccinated with LNP, LGP, EGP (the EGP results were described in the above VEE-replicon-vectored EBOV vaccine section), a mixture of LNP+LGP or LGP+EGP, or with a double promoter replicon expressing both LGP and EGP (LGP/EGP). Passive serum transfer studies revealed that serum transferred from guinea pigs vaccinated with VEE-replicons expressing either LGP or LNP to naïve guinea pigs did not passively protect the animals from challenge. Whereas, guinea pigs inoculated with replicon expressing LNP, LGP, or a mixture of LNP and LGP were completely protected from challenge. This result suggests that a cellular immune response is necessary to protect against Lassa fever. Additional studies involving adoptive transfer of CTLs may help in determining the mechanism of immune protection against Lassa fever. To evaluate the efficacy of a combined EBOV and LSV vaccine, guinea pigs inoculated with LGP+EGP or LGP/EGP were challenged with either guinea pig-adapted EBOV or LS. The LGP+EGP vaccine protected four out of five animals (no viremia was detected in the animal that died on day 14 after challenge) from a LSV challenge and five of five animals from an EBOV challenge. The LGP/EGP vaccine protected five of five animals from a LSV challenge and three of five animals (no viremia and no disease was detected in the two animals that died on day 21 or 29 after challenge) from an EBOV challenge. As expected, the replicon expressing EGP did not protect the guinea pigs from a LSV challenge and conversely, LGP did not protect the animals from an EBOV challenge. Additional studies elucidating the mechanism of protection elicited by the mixed replicon and the double-promoter replicon vaccines may help in defining a potential role of this approach for use in humans.

3.2.3. VEEV-vectored staphylococcal enterotoxin B vaccines

Numerous cases of food poisoning and hospital-acquired infections are caused by *Staphylococcus aureus* bacteria. Exotoxins produced by the organism, referred to as enterotoxins because they exert their effects on the gastrointestinal tract, cause severe gastrointestinal distress, diarrhea and vomiting, and are also a cause of potentially lethal toxic shock syndrome. These enterotoxins also act as super-antigens and cause disease by binding to major histocompatibility complex class II molecules on antigen-presenting cells and T-cell antigen receptors resulting in the release of large amounts of proinflammatory cytokines [85,86]. Staphylococcal enterotoxin B (SEB) was weaponized by the U.S. before the late 1960s [1]. The relative stability of SEB in aerosols and the ability of SEB aerosols to incapacitate humans many miles downwind of a release site made this an ideal agent for biowarfare development [22]. Only very low doses of SEB are necessary to cause symptoms in humans resulting in clinical presentation of SEB intoxication within hours of exposure. NIAID has classified SEB as a category B pathogen and no licensed vaccine currently exists for protection against SEB.

An effective vaccine against SEB was constructed by cloning a mutagenized SEB gene (mSEB) into the VEE-replicon vector [87]. The mSEB gene contained three mutations that abolished its ability to interact with T-cells thus preventing it from causing disease [88]. Mice inoculated with VEE-replicon expressing mSEB were partially protected (15 of 20 mice) from a lethal challenge with wild-type SE (wt-SEB). The same level of protection was observed in control mice inoculated with recombinant mSEB protein combined with aluminum hydroxide (15 of 19 mice survived challenge). The profile of cytokines measured after wt-SEB challenge suggested that the mode of protection resulting from VEEV-replicon-based vaccination was predominantly Th1-dependent. Even though the mice mounted a cellular immune response, they also responded with an antibody response that provided protection from the effects of the wt-SEB challenge. These results suggest that VEE-replicons

are effective at stimulating cellular and humoral immune responses in this model.

3.2.4. VEEV-vectored botulinum neurotoxin vaccines

BoNT was produced and weaponized by the U.S. before the BWC. Iraq admitted to United Nations inspection teams of having performed research in producing and weaponizing BoNT before the Persian Gulf and were reported to have filled approximately 100 munitions containing BoNT [1]. Because BoNTs are the most toxic compounds known with an estimated toxic dose of 1 ng/kg of body weight, NIAID has classified the BoNTs as category A pathogens. The anaerobic bacterium *Clostridium botulinum* produces one or more of the seven distinct serotypes of BoNT depending on the strain of *C. botulinum*. All seven serotypes of BoNT act by similar mechanisms to induce a flaccid paralysis when inhaled or ingested [89,90]. BoNTs consist of two polypeptides, a heavy chain of about 100 kDa and a light chain of about 50 kDa, bound by a disulfide bond. Botulism, the disease caused BoNT intoxication, usually results from the consumption of improperly prepared or canned foods. Previous research has shown that polyclonal antibodies to one serotype can only block the effects of the homologous serotype [91]. The current human vaccine, which is administered under Investigational New Drug status to at-risk laboratory personnel, contains five of the seven serotypes (A-E). The toxoid vaccine is given as a primary series of three inoculations given at 0, 2, and 12 weeks, followed by a booster at 1 year and is reactogenic in up to 20% of the recipients. The vaccine is expensive to manufacture and production of the large amounts of active toxin necessary for the toxoiding process poses safety and security issues.

A candidate vaccine against BoNT serotype A (BoNT/A) was developed by cloning the non-toxic 50-kDa carboxy-terminal fragment (Hc) from the heavy chain of BoNT/A (BoNT/A Hc) into the VEEV-replicon vector [92]. Vaccinated mice were protected from an i.p. challenge of up to 100,000 LD_{50} units of BoNT/A and protection correlated directly with serum ELISA titers to BoNT/A. The mice maintained high circulating antibody levels and remained refractory to challenge with BoNT/A at both 6 months and 12 months post-vaccination. These results demonstrate that the VEEV-replicon is capable of eliciting long-lasting antibody responses in animals.

3.2.5. VEEV-vectored anthrax vaccines

The VEEV-replicon has proven to be a very effective tool for constructing vaccines against diverse organisms and toxins. Three different anthrax vaccines were constructed by cloning the PA gene from *B. anthracis* into the VEEV-replicon vector [93]. Mice inoculated with the mature 83-kDa PA vaccine (MAT-PA) were completely protected from challenge with the Sterne strain of *B. anthracis*. Similar results were obtained with vaccines composed of the PA gene fused to either the *B. anthracis* secretory sequence (b-PA) or to a tissue plasminogen activator secretory sequence (TPA-PA) in mice. Because the VEEV-replicon did not produce antibody titers in mice as high as AVA (the vaccine used in humans), we are currently developing ways of improving expression and delivery of the replicons. For example, improvements that are already underway include changing the native PA gene sequence, which contains approximately 70% adenosine or thymidine, to a synthetic gene sequence that encodes for codons more frequently used by mammalian cells. The different VEEV-replicon vaccines expressing PA were effective at protecting animals against anthrax with as few as two doses without the need for adjuvants or formulations with formaldehyde.

3.3. Semliki Forest virus-vectored vaccines against Puumala virus

Semliki Forest virus (SFV) has been used to express a wide variety of genes for gene therapy and for vaccine applications [94]. Assembly of SFV-replicon into VLP for inoculation into animals was similar to the single-helper RNA method used for SINV-replicons but was found not to produce any replication-competent virus [95]. Expression of the NP gene of PUUV (Bunyaviridae family and a NIAID category A pathogen as described above) by SFV-replicon (SFV-Npuu) produced protein that was indistinguishable from native protein derived from PUUV-infected cells. The goal of the study was to produce protein that could be used in the serological diagnosis of PUUV infection in northern and central Europe. Even though the authors did not vaccinate

any animals in this study, the potential of using SFV-Npuu as an effective vaccine is promising. This study is very similar to the study described in Section 3.1.3 above except the SINV-replicon vector was used to elicit antibodies specific to SEOV in the vaccinated mice. One advantage of using the SFV-replicon is that cells infected with the replicon express the foreign protein for up to 75 h [95]. The prolonged expression may increase the immunogenicity and efficacy of vaccines that use this vector.

4. Adenovirus-vectored vaccines against Ebola hemorrhagic fever

Adenovirus (Ad) was first discovered in 1954 when scientists were trying to establish cells lines derived from tonsil and adenoidal tissues and has recently been utilized extensively as a vector for gene therapy [96,97]. Human Ad belongs to the Adenoviridae family which is composed of two genera [98]. The genus *Mastadenovirus* contains 51 human Ad types, along with other mammalian Ad types. The linear double-stranded DNA genome of Ad (36 kbp for Ad type 2, or Ad2) allows for insertion of vaccine genes of up to 8 kbp without disrupting viral replication or packaging. Genes larger than 8 kbp can be inserted by deleting an equivalent part of the viral genome. For vaccine purposes, the E1 gene required for viral replication was removed and a packaging system was created to produce replication-defective Ad containing the foreign gene of interest [99].

A combination of plasmids and Ad-expressed EBOV proteins was used in a prime-boost method of vaccination designed to protect animals from EBOV [100]. The prime-boost method of vaccination in mice involved a primary inoculation with either plasmid expressing EBOV GP Zaire strain, pGP(Z), or with Ad5 expressing the same protein, ADV-GP(Z), followed by a secondary inoculation with either the homologous vaccine or with the heterologous vaccine. Mice produced the highest antibody responses when inoculated with pGP(Z) followed by inoculation with ADV-GP(Z). The antibody response in those animals was approximately 2-fold higher than that determined in animals vaccinated with ADV-GP(Z) followed by ADV-GP(Z) and about 10-fold higher than that determined in animals vaccinated with pGP(Z) followed by pGP(Z). The opposite vaccination strategy of ADV-GP(Z) followed by pGP(Z) was not reported. A similar prime-boost vaccination strategy was also used to vaccinate NHPs against EBOV. The NHPs were inoculated with four plasmids, three expressing different types of EBOV GP and one plasmid expressing NP from EBOV(Z), on weeks 0, 4, and 8 followed by inoculation with ADV-GP(Z) on week 20. Moderate enzyme-linked immunosorbent assay (ELISA) antibody titers of approximately 3 logs were determined for the NHPs at week 12 which increased significantly to approximately 4.5 to 5 logs after the booster vaccination. Challenge of the NHPs at week 32 with a low challenge dose of 6 PFU of EBOV(Z) resulted in the survival of all of the animals. The challenge dose was not great enough to conclusively determine the effectiveness of the vaccination strategy and the protection it afforded the animals. The use of a prime-boost strategy for developing an EBOV vaccine may translate into an effective method for vaccinating humans against EBOV. Additional studies in NHP with higher challenge levels will determine the efficacy of such vaccines.

5. Other viral vectors that may have utility in biodefense vaccine development

Several other viral vectors have been constructed that may have application in the development of biodefense vaccines. A poliovirus-vectored vaccine against chicken ovalbumin (OVA) was constructed and found to elicit specific antibody responses against OVA in naïve and in poliovirus immune mice [101]. The only adverse result was that the CTL-induced responses were reduced in the poliovirus immune mice. Vectors that display resistance to preexisting immunity might be useful as vaccine vectors for individuals that require multiple vaccinations. Mengo virus, a picornavirus, has also been used in the development of vaccines. Mengo virus-vectored lymphocytic choriomeningitis virus (LCMV) vaccine was shown to completely protect mice from a LCMV challenge [102]. This vector was also effective for induction of CTL responses in the

vaccinated mice. These picornavirus-vectored vaccines offer the potential for oral administration thus reducing the problems associated with needle-type inoculations.

Kunjin (KUN) and yellow fever (YF) virus, both flaviviruses, have also been engineered to express vaccine-related proteins or T-cell epitopes. The KUN vaccine vector is non-cytopathic and directed prolonged expression of heterologous genes in cultured cells [103]. Vaccinating mice with the KUN vector expressing murine polyepitope stimulated protective CTL responses in the mice against a recombinant VV or against a B16-OVA tumor challenge [104]. Similar to KUN, YF virus engineered to express OVA was able to elicit protective CTL responses in vaccinated mice against a lethal challenge with malignant melanoma cells expressing OVA [105]. The flavivirus vaccine vectors represent a class of vectors that could be useful against diseases that require CTL responses to neutralize the virus- or bacteria-infected host cells.

Recent development of additional viral vectors has expanded the possible choices for constructing new biodefense vaccines. Mouse hepatitis virus, a member of the Coronaviridae family, was used to construct a replication-competent vector that efficiently expressed two different luciferase genes in cultured cells [106]. Rabies virus (RV) and vesicular stomatitis virus (VSV), members of the Rhabdoviridae family, were also used to construct live-attenuated vectors that were useful at stimulating immune responses in animals against human immunodeficiency virus (HIV) [107,108]. One advantage of using RV as a vector is that it allows for expression of multiple genes and of very large genes (approximately 6.6 kb). Vaccine vectors constructed from two members of the Paramyxoviridae family, Sendai virus and bovine parainfluenza virus type 3, have been effective at inducing protective immune responses against simian-HIV and against respiratory syncytial virus in animals, respectively [109,110]. Because different viruses display different cellular tropisms and can stimulate different types of immune responses, constructing viral vectors based on these different viruses maybe useful in designing new vaccines that can stimulate immune responses most appropriate for a given biological threat agents.

6. Anti-vector immune responses associated with virus-vectored vaccines

Anti-vector immune responses are a concern for at-risk personnel (to include first-responders, the military, and scientists working with the agents) as they may require vaccinations to multiple pathogens and toxins. The repeated use of the same viral vector may be hampered by anti-vector immune responses in the vaccine recipient. Sequential vaccinations against EBOV, BoNT, and anthrax may not be possible with all of the vectors mentioned above. Immune responses against VV have hampered the use of this virus for multiple inoculations. Previous research has shown that animals vaccinated with VV- or Ad-vectored vaccines often develop high neutralizing responses against the vector as well as immune responses against the foreign gene [99,111,112]. The high neutralizing anti-vector responses then prevent the vaccine recipient from responding to a second vaccine vectored by the same virus. Phase I and Phase II clinical trials of VACV, the VV-vectored HTNV vaccine, also showed similar results [51]. Pre-existing VV immunity interfered with the ability of VACV to stimulating immune responses to HTNV in vaccinated individuals. Of the 43 VV-naïve and the 47 VV-preimmune individuals inoculated with two doses of VACV, 72% of the VV-naïve individuals responded with neutralizing antibody responses to HTNV as compared to only 26% of the VV-preimmune individuals. One dose or two doses of VACV stimulated anti-VV responses in 67% or 98% of the vaccinated individuals, respectively, and shows how immunogenic VV vectors can be. The recent development of a recombinant modified VV Ankara (rMVA) may alleviate some of the anti-VV vector immunity problems and may allow for sequential vaccinations against heterologous pathogens using the same vector and for vaccination of persons recently vaccinated against smallpox [113]. Studies evaluating the route of Ad-vectored vaccine inoculation found that intramuscular inoculation prevented mice from responding to a second intramuscular vaccination with the same vector but did not prevent the mice from responding to the same vector when inoculated orally [114]. Oral inoculation with the Ad-vectored vaccines does offer the advantage of needle-free delivery but the immuno-

genicity of the vaccines when given orally, with or without preexisting immunity to Ad, was greatly diminished. The use of different Ad types with minimal preexisting immunity in the human population (e.g., Ad35) [99] and using different animal species of Ad [115] for each sequential inoculation may prove useful in the long-term use of Ad as a vaccine vector.

The repeated use of alphavirus vectors for vaccinating animals has not produced the same anti-vector effects in animals as has VV and Ad. Sequential inoculations with two different VEEV-replicons expressing LSN followed by influenza HA protein elicited antibody responses in mice to both proteins [75]. The antibody responses and protection were not diminished following sequential vaccination and no anti-VEEV antibody responses were detected in the BALB/c mice used in the experiment. In contrast, Swiss mice inoculated with a different VEEV-vectored vaccine did produce neutralizing antibodies against VEEV but the neutralizing antibodies did not interfere with subsequent booster vaccinations [92]. Sequential inoculation of animals with SINV-replicons expressing SEOV-S then with SINV-replicons expressing the lacZ gene elicited antibodies to each protein, although antibody levels were lower in the mice with preexisting anti-SINV antibodies [73]. The generation of anti-SINV antibodies in the animals was probably due to the presence of replication-competent virus that was produced during the VLP assembly. Use of a bipartite helper system for the assembly of VLPs, similar to the one used for VRP assembly, eliminates the regeneration of replication-competent virus. The limit on the number of alphavirus-vectored inoculations that can be administered before anti-vector responses prevent effective vaccination has not been determined. Additional studies will determine if alphavirus vectors can be administered multiple times, even in the presence of neutralizing antibodies, to protect humans against multiple biological warfare agents.

7. Perspectives

A wide array of viral vectors have been constructed and used to develop vaccines against biowarfare and bioterrorism agents. The ability to obtain pathogens from the environment and the ease at which terrorist groups can prepare large quantities of these pathogens drives the development of biodefense vaccines. Because some of the pathogens cause natural outbreaks, constructing the biodefense vaccines may also help nations fight endemic diseases afflicting their respective countries. Because bioterrorism is not limited to attacks against human populations, the same viral vectors that were used to make vaccines for use in humans can easily be converted into vaccines for use in economically important animals such as dairy cows, beef cattle, swine, and chickens. Agricultural bioterrorism was not discussed in this review, but the vaccine vectors discussed here may have application for protecting animals from bioterrorism.

Viral vectors offer the advantage of a rapid method for preparing vaccine without the need for purifying proteins or preparing adjuvant. The continued development of vaccine vectors that target specific immune effector cells may lead to more immunogenic and protective vaccines. Even though some of the viral vectors mentioned above stimulate neutralizing immune responses, constructing new vectors that are resistant to neutralization or that use different serotypes of the same virus may permit sequential inoculation of different vaccines into the same individual. Combining virus-vectored vaccines with other types of vaccines, e.g., DNA vaccines and recombinant protein with adjuvant vaccines, may increase their overall efficacy by stimulating better immune responses than either of the individual components. This review reports the feasibility of using recombinant viruses and viral vectors in the development of biodefense vaccines. The development and use of vaccines against agents used in biowarfare and bioterrorism may help dissuade individuals and rogue nations from using biological agents in acts of terrorism or on the battlefield.

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References

[1] G.W. Christopher, T.J. Cieslak, J.A. Pavlin, E.M. Eitzen, Biological warfare: a historical perspective, J. Am. Med. Assoc. 278 (1997) 412–417.

[2] R.P. Kadlec, A.P. Zelicoff, A.M. Vrtis, Biological weapons control: prospects and implications for the future, J. Am. Med. Assoc. 278 (1997) 351–356.

[3] R.A. Zilinskas, Iraq's biological weapons, J. Am. Med. Assoc. 278 (1997) 418–424.

[4] S. McCall, A higher form of killing, Proc. US Naval. Inst. 121 (1995) 40–45.

[5] Investigation of bioterrorism-related anthrax: Connecticut, Morb. Mortal. Wkly. Rep., CDC Surveill. Summ. 50 (2001) 1077–1079.

[6] S.B. Halstead, J. Deen, The future of dengue vaccines, Lancet 360 (2002) 1243–1245.

[7] J. Donnelly, K. Berry, J.B. Ulmer, Technical and regulatory hurdles for DNA vaccines, Int. J. Parasitol. 33 (2003) 457–467.

[8] F.R. Vogel, Improving vaccine performance with adjuvants, Clin. Infect. Dis. 30 (2000) S266–S270.

[9] S. Liljeqvist, S. Stahl, Production of recombinant subunit vaccines: protein immunogens, live delivery systems and nucleic acid vaccines, J. Biotechnol. 73 (1999) 1–33.

[10] T.O. Berge, I.S. Banks, W.D. Tigertt, Attenuation of Venezuelan equine encephalomyelitis virus by in vitro cultivation in guinea pig heart cells, Am. J. Hyg. 73 (1961) 209–218.

[11] R.W. McKinney, T.O. Berge, W.D. Sawyer, W.D. Tigertt, D. Crozier, Use of attenuated strain of Venezuelan equine encephalitis virus for immunization in man, Am. J. Trop. Med. Hyg. 12 (1963) 597–603.

[12] D.S. Burke, Immunization against tularemia, J. Infect. Dis. 135 (1977) 55–60.

[13] A.J. Macadam, G. Ferguson, D.M. Stone, J. Meredith, J.W. Almond, P.D. Minor, Live-attenuated strains of improved genetic stability, Dev. Biol. (Basel) 105 (2001) 179–187.

[14] H.S. Garmony, R.W. Titball, K.F. Griffin, U. Hahn, R. Bohm, W. Beyer, *Salmonella enterica* serovar *typhimurium* expressing a chromosomally integrated copy of *Bacillus anthracis* protective antigen gene protects mice against an anthrax spore challenge, Infect. Immun. 71 (2003) 3831–3836.

[15] H.S. Garmony, K.F. Griffin, K.A. Brown, R.W. Titball, Oral immunization with live aroa attenuated *Salmonella enterica* serovar *typhimurium* expressing the *Yersinia pestis* V antigen protects mice against plague, Vaccine 20 (2003) 3051–3057.

[16] B. Moss, Poxviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 2, 2001, pp. 2849–2883.

[17] B. Moss, Genetically engineered poxviruses for recombinant gene expression, vaccination, and safety, Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11341–11348.

[18] K. Aspden, J.A. Passmore, F. Tiedt, A.L. Willianson, Evaluation of lumpy skin disease virus, a *Capripoxvirus*, as a replication-deficient vaccine vector, J. Gen. Virol. 84 (2003) 1985–1996.

[19] T. Fischer, O. Planz, L. Stitz, H.-J. Rziha, Novel recombinant parapoxvirus vectors induce protective humoral and cellular immunity against lethal herpesvirus challenge infection in mice, J. Virol. 77 (2003) 9312–9323.

[20] A. Radaelli, J. Nacsa, W.P. Tsai, Y. Edghill-Smith, C. Zanotto, V. Elli, D. Venzon, E. Tryniszewska, P. Markham, G.P. Mazzara, D. Panicali, C. de giuli Morghen, G. Franchini, Prior DNA immunization enhances immune responses to dominant and subdominant viral epitopes induced by a fowlpox-based Sivmac vaccine in long-term slow-progressor macaques infected with Sivmac251, Virology 312 (2003) 181–195.

[21] L. Borio, T.V. Inglesby, C.J. Peters, A. Schmaljohn, J. Hughes, P. Jahrling, T.G. Ksiazek, K.M. Johnson, A. Meyerhoff, T. O'Toole, M.S. Ascher, J. Bartlett, J.G. Breman, E.M. Eitzen Jr., M. Hamburg, J. Hauer, D.A. Henderson, R.T. Johnson, G. Kwik, M. Layton, S. Lillibridge, G.J. Nabel, M.T. Osterholm, T.M. Perl, P.K. Russell, K. Tonat, Hemorrhagic fever viruses as biological weapons, J. Am. Med. Assoc. 287 (2002) 2391–2405.

[22] D.R. Franz, P.B. Jahrling, A.M. Friedlander, D.J. McClain, D.L. Hoover, W.R. Bryne, J.A. Pavlin, G.W. Christopher, E.M. Eitzen Jr., Clinical recognition and management of patients exposed to biological warfare agents, J. Am. Med. Assoc. 278 (1997) 399–411.

[23] M.J. Buchmeier, M.D. Bowen, C.J. Peters, Arenaviridae: the viruses and their replication, in: D.M. Knipe, J.W. Hooper (Eds.), Fields Virology, vol. 2, 2001, pp. 1635–1668.

[24] J.D. Frame, J.M.J. Baldwin, D.J. Gocke, J.M. Troup, Lassa fever, a new virus disease of man from west Africa: I. Clinical description and pathological finding, Am. J. Trop. Med. Hyg. 19 (1970) 670–676.

[25] D.D. Auperin, J.J. Esposito, J.V. Lange, S.P. Bauer, J. Knight, D.R. Sasso, J.B. McCormick, Construction of a recombinant vaccinia virus expressing the Lassa virus glycoprotein gene and protection of guinea pigs from a lethal Lassa virus infection, Virus Res. 9 (1988) 233–248.

[26] J.C.S. Clegg, G. Lloyd, Vaccinia recombinant expressing Lassa-virus internal nucleocapsid protein protects guinea pigs against Lassa fever, Lancet ii (1987) 186–188.

[27] H.G. Morrison, S.P. Bauer, J.V. Lange, J.J. Esposito, J.B. McCormick, D.D. Auperin, Protection of guinea pigs from Lassa fever by vaccinia virus recombinants expressing the nucleoprotein or the envelope glycoproteins of Lassa virus, Virology 171 (1989) 179–188.

[28] H.G. Morrison, C.S. Goldsmith, H.L. Regnery, D.D. Auperin, Simultaneous expression of the Lassa virus N and Gpc genes from a single recombinant vaccinia virus, Virus Res. 18 (1990) 231–242.

[29] S.P. Fisher-Hoch, J.B. McCormick, D.D. Auperin, B.G. Brown, M. Castor, G. Perez, S.L. Ruo, A. Conaty, L. Brammer, S.P. Bauer, Protection of rhesus monkeys from fatal Lassa fever by vaccination with a recombinant vaccinia virus containing the Lassa virus glycoprotein gene, Proc. Natl. Acad. Sci. U. S. A. 86 (1989) 317–321.

[30] S.P. Fisher-Hoch, L. Hutwagner, B. Brown, J.B. McCormick, Effective vaccine for Lassa fever, J. Virol. 74 (2000) 6777–6783.
[31] A. Sanchez, A.S. Khan, S.R. Zaki, G.J. Nabel, T.G. Ksiazek, C.J. Peters, *Filoviridae*: Marburg and Ebola viruses, in: D.M. Knipe, P.M. Howley, D.E. Griffin, M.A. Martin, R.A. Lamb, B. Roizman, et al. (Eds.), Fields Virology, 2001, pp. 1279–1304.
[32] J.K. Gilligan, J.B. Geisbert, P.B. Jahrling, K. Anderson, Assessment of protective immunity conferred by recombinant VACV to guinea pigs challenged with Ebola virus, in: F. Brown, D. Burton, P. Doherty, J. Mekalanos, E. Norrby (Eds.), Vaccines, 1997, pp. 87–92.
[33] J.K. Gilligan, J. Geisbert, P. Jahrling, K. Anderson, Assessment of protective immunity conferred by recombinant vaccinia viruses to guinea pigs challenged with Ebola virus, in: F. Brown, D. Burton, P. Doherty, J. Mekalanos, E. Norrby (Eds.), Vaccines, vol. 97, 1997, pp. 87–92.
[34] T.W. Geisbert, P. Pushko, K. Anderson, J.F. Smith, K.J. Davis, P.B. Jahrling, Evaluation in nonhuman primates of vaccines against Ebola virus, Emerg. Infect. Dis. 8 (2002) 503–507.
[35] R. Rico-Hesse, Venezuelan equine encephalomyelitis, Emerg. Infect. Dis. 16 (2000) 553–563.
[36] D.E. Griffin, Alphaviruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 1, 2001, pp. 917–962.
[37] S.C. Weaver, R. Salas, R. Rico-Hesse, G.V. Ludwig, M.S. Oberste, J. Boshell, R.B. Tesh, Re-emergence of epidemic Venezuelan equine encephalomyelitis in South America, Lancet 348 (1996) 436–440.
[38] A.C. Alevizatos, R.W. McKinney, R.D. Feigin, Live, attenuated Venezuelan equine encephalomyelitis virus vaccine: I. Clinical effects in man, Am. J. Trop. Med. Hyg. 16 (1967) 762–768.
[39] W.D. Pratt, N.L. Davis, R.E. Johnston, J.F. Smith, Genetically engineered, live attenuated vaccine for Venezuelan equine encephalitis: testing in animal models, Vaccine 21 (2003) 3854–3862.
[40] R.M. Kinney, J.J. Esposito, J.H. Mathews, B.J.B. Johnson, J.T. Roehrig, A.D.T. Barrett, D.W. Trent, Recombinant vaccinia virus/Venezuelan equine encephalitis (Vee) virus protects mice from peripheral Vee virus challenge, J. Virol. 62 (1988) 4697–4702.
[41] R.J. Phillpotts, T.L. Lescott, S.C. Jacobs, Vaccinia virus recombinants encoding the truncated structural gene region of Venezuelan equine encephalitis virus (Veev) give solid protection against perpheral challenge but only partial protection against airborne challenge with virulent VEEV, Acta Virol. 44 (2000) 233–239.
[42] A.M. Bennett, T. Lescott, R.J. Phillpotts, Improved protection against Venezuelan equine encephalitis by genetic engineering of a recombinant vaccinia virus, Viral Immunol. 11 (1998) 109–117.
[43] S.T. Nichol, Bunyaviruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 2, 2001, pp. 1603–1633.
[44] C.S. Schmaljohn, B. Hjelle, Hanntaviruses: a global disease problem, Emerg. Infect. Dis. 3 (1997) 95–104.
[45] C.S. Schmaljohn, J.W. Hooper, Bunyaviridae: the viruses and their replication, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 2, 2001, pp. 1581–1602.
[46] Y.M. Sohn, H.O. Rho, M.S. Park, J.S. Kim, P.L. Summers, Primary humoral immune responses to formalin inactivated hemorrhagic fever with renal syndrome vaccine (Hantavax): consideration of active immunization in South Korea, Yonsei, Med. J. 42 (2001) 278–284.
[47] C.S. Schmaljohn, Y.-K. Chu, A. Schmaljohn, J.M. Dalrymple, Antigenic subunits of Hantaan virus expressed by baculovirus and vaccinia virus recombinants, J. Virol. 64 (1990) 3162–3170.
[48] C.S. Schmaljohn, S.E. Hasty, J.M. Dalrymple, Preparation of candidate vaccinia-vectored vaccines for haemorrhagic fever with renal syndrome, Vaccine 10 (1992) 10–13.
[49] Y.K. Chu, G.B. Jennings, C.S. Schmaljohn, A vaccinia virus-vectored Hantaan virus vaccine protects hamsters from challenge with Hantaan and Seoul viruses but not Puumala virus, J. Virol. 69 (1995) 6417–6423.
[50] X. Xu, S.L. Ruo, J.B. McCormick, S.P. Fisher-Hoch, Immunity to hantavirus challenge in *Meriones unguiculatus* induced by vaccinia-vectored viral proteins, Am. J. Trop. Med. Hyg. 47 (1992) 397–404.
[51] D.J. McClain, P.L. Summers, S.A. Harrison, A. Schmaljohn, C.S. Schmaljohn, Clinical evaluation of a vaccinia-vectored Hantaan virus vaccine, J. Med. Virol. 60 (2000) 77–85.
[52] T.V. Inglesby, T. O'Toole, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E. Eitzen, A. Friedlander, J. Gerberding, J. Hauer, J. Hughes, J. McDade, M.T. Osterholm, G. Parker, T.M. Perl, P.K. Russell, K. Tonat, Anthrax as a biological weapon, 2002: updated recommendations for management, J. Am. Med. Assoc. 287 (2002) 2236–2252.
[53] M. Meselson, J. Guillemin, M. Hugh-Jones, A. Langmuir, I. Popova, A. Shelokov, O. Yampolskaya, The Sverdlovsk anthrax outbreak of 1979, Science 266 (1994) 1202–1208.
[54] M. Kortepeter, G.W. Christopher, T.J. Cieslak, R. Culpepper, R. Darling, J.A. Pavlin, J. Rowe, S. Stanek, E.M. Eitzen Jr., K. McKee Jr., USAMRIID's Medical Management of Biological Casualties Handbook, 2001, p. 165.
[55] L.C. Iacono-Connors, C.S. Schmaljohn, J.M. Dalrymple, Expression of the *Bacillus anthracis* protective antigen gene by baculovirus and vaccinia virus recombinants, Infect. Immun. 58 (1990) 366–372.
[56] L.C. Iacono-Connors, S.L. Welkos, B.E. Ivins, J.M. Dalrymple, Protection against anthrax with recombinant virus-expressed protective antigen in experimental animals, Infect. Immun. 59 (1991) 1961–1965.
[57] M.L.M. Pitt, S. Little, B. Ivins, P. Fellows, J. Barth, J. Hewetson, P. Gibbs, M. Dertzbaugh, A. Friedlander, In vitro correlate of immunity in a rabbit model of inhalation anthrax, Vaccine 19 (2001) 4768–4773.
[58] J. Ko, G.A. Splitter, Molecular host–pathogen interaction in brucellosis: current understanding and future approaches to vaccine development for mice and humans, Clin. Microbiol. Rev. 16 (2003) 65–78.
[59] D.L. Hoover, A.M. Friedlander, Brucellosis, in: R. Zajtchuk, R.F. Bellamy (Eds.), Textbook of Military Medicine:

Medical Aspects of Chemical and Biological Warfare, 1997, pp. 513–521.
[60] L.A. Guilloteau, K. Laroucau, N. Vizcaino, I. Jacques, G. Dubray, Immunogenicity of recombinant *Escherichia coli* expressing OMP31 gene of *Brucella melitensis* in Balb/C mice, Vaccine 17 (1999) 353–361.
[61] P.A. Denoel, T.K. Vo, V.E. Weynants, A. Tibor, D. Gilson, M.S. Zygment, J.N. Limet, J.J. Letesson, Identification of the major T-cell antigens present in the *Brucella melitensis* B115 protein preparation, Brucellergene Ocb, J. Med. Microbiol. 46 (1997) 801–806.
[62] A. Tibor, I. Jacques, L.A. Guilloteau, J.M. Verger, M. Grayon, V. Wansard, J.J. Letesson, Effect of the P39 gene deletion in live *Brucella* vaccine strains on residual virulence and protective activity in mice, Infect. Immun. 66 (1998) 5561–5564.
[63] T.V. Inglesby, D.T. Dennis, D.A. Henderson, J.G. Bartlett, M.S. Ascher, E.M. Eitzen Jr., A.D. Fine, A.M. Friedlander, J. Hauer, J.F. Koerner, M. Layton, J. McDade, M.T. Osterholm, T. O'Toole, G. Parker, T.M. Perl, P.K. Russell, M. Schoch-Spana, K. Tonat, Plague as a biological weapon, J. Am. Med. Assoc. 283 (2000) 2281–2290.
[64] J.E. Osorio, T.D. Powell, R.S. Frank, K. Moss, E.J. Haanes, S.R. Smith, T.E. Rocke, D.T. Stinchcomb, Recombinant raccoon pox vaccine protects mice against lethal plague, Vaccine 21 (2003) 1232–1238.
[65] J.H. Strauss, E.G. Strauss, The alphaviruses: gene expression, replication, and evolution, Microbiol. Rev. 58 (1994) 491–562.
[66] S. Schlesinger, Alphavirus expression vectors, Adv. Virus Res. 55 (2000) 565–577.
[67] P.J. Bredenbeek, I. Frolov, C.M. Rice, S. Schlesinger, Sindbis virus expression vectors: packaging of Rna replicons by using defective helper Rnas, J. Virol. 67 (1993) 6439–6446.
[68] R. Raju, S.V. Subramaniam, M. Hajjou, Genesis of Sindbis virus by in vivo recombination of nonreplicative Rna precursors, J. Virol. 69 (1995) 7391–7401.
[69] I. Frolov, E. Frolov, S. Schlesinger, Sindbis virus replicons and Sindbis virus: assembly of chimeras and of particles deficient in virus Rna, J. Virol. 71 (1997) 2819–2829.
[70] C. Xiong, R. Levis, P. Shen, S. Schlesinger, C.M. Rice, H.V. Huang, Sindbis virus: an efficient, broad host range vector for gene expression in animal cells, Science 243 (1989) 1188–1191.
[71] S.D. London, A.L. Schmaljohn, J.M. Dalrymple, C.M. Rice, Infectious enveloped Rna virus antigenic chimeras, Proc. Natl. Acad. Sci. U. S. A. 89 (1992) 207–211.
[72] S. Paessler, R.Z. Fayzulin, M. Anishchenko, I.P. Greene, S.C. Weaver, I. Frolov, Recombinant Sindbis/Venezuelan equine encephalitis virus is highly attenuated and immunogenic, J. Virol. 77 (2003) 9278–9286.
[73] K.I. Kamrud, J.W. Hooper, F. Elgh, C.S. Schmaljohn, Comparison of the protective efficacy of naked DNA, DNA-based Sindbis replicon, and packaged Sindbis replicon vectors expressing hantavirus structural genes in hamsters, Virology 263 (1999) 209–219.
[74] D.M. Custer, E. Thompson, C.S. Schmaljohn, T.G. Ksiazek, J.W. Hooper, Active and passive vaccination against hantavirus pulmonary syndrome with Andes virus M genome segment-based DNA vaccine, J. Virol. 77 (2003) 9894–9905.
[75] P. Pushko, M. Parker, G.V. Ludwig, N.L. Davis, R.E. Johnston, J.F. Smith, Replicon–helper systems from attenuated Venezuelan equine encephalitis virus: expression of heterologous genes in vitro and immunization against heterologous pathogens in vivo, Virology 239 (1997) 389–401.
[76] G.H. MacDonald, R.E. Johnston, Role of dendritic cell targeting in Venezuelan equine encephalitis virus pathogenesis, J. Virol. 74 (2000) 914–922.
[77] S. Perri, C.E. Greer, K. Thudium, B. Doe, H. Legg, H. Liu, R.E. Romero, Z. Tang, Q. Bin, T.W. Dubensky Jr., M. Vajdy, G.R. Otten, J.M. Polo, An alphavirus replicon particle chimera derived from Venezuelan equine encephalitis and Sindbis viruses is a potent gene-based vaccine delivery vector, J. Virol. 77 (2003) 10394–10403.
[78] J.A. Wilson, M. Bray, R. Bakken, M.K. Hart, Vaccine potential of Ebola virus Vp24, Vp30, Vp35, and Vp40 proteins, Virology 286 (2001) 384–390.
[79] J.A. Wilson, M.K. Hart, Protection from Ebola virus mediated by cytotoxic T lymphocytes specific for the viral nucleoprotein, J. Virol. 75 (2001) 2660–2664.
[80] P. Pushko, M. Bray, G.V. Ludwig, M. Parker, A. Schmaljohn, A. Sanchez, P. Jahrling, J.F. Smith, Recombinant Rna replicons derived from attenuated Venezuelan equine encephalitis virus protect guinea pigs and mice from Ebola hemorrhagic fever virus, Vaccine 19 (2001) 142–153.
[81] P. Pushko, M. Parker, J. Geisbert, D. Negley, A. Schmaljohn, P. Jahrling, J. Smith, Venezuelan equine encephalitis virus replicon vector: immunogenicity studies with Ebola Np and Gp genes in guinea pigs, in: F. Brown, D. Burton, P. Doherty, J. Mekalanos, E. Norrby (Eds.), Journal/Vaccines, vol. 97, 1997.
[82] P. Pushko, J. Geisbert, M. Parker, P. Jahrling, J.F. Smith, Individual and bivalent vaccines based on alphavirus replicons protect guinea pigs against infection with Lassa and Ebola viruses, J. Virol. 75 (2001) 11677–11685.
[83] C.J. Peters, A. Sanchez, P.E. Rollin, T.G. Ksiazek, F.A. Murphy, *Filoviridae*: Marburg and Ebola viruses, in: B.N. Fields, D.M. Knipe, P.M. Howley (Eds.), Fields Virology, 1996, pp. 1161–1176.
[84] M. Hevey, D. Negley, P. Pushko, J. Smith, A. Schmaljohn, Marburg virus vaccines based upon alphavirus replicons protect guinea pigs and nonhuman primates, Virology 251 (1998) 28–37.
[85] R.G. Ulrich, S. Bavari, M.A. Olson, Staphylococcal enterotoxin a and b share a common structural motif for binding class Ii major histocompatibility complex molecules, Nat. Struct. Biol. 2 (1995) 554–560.
[86] R.G. Ulrich, S. Bavari, M.A. Olson, Bacterial superantigens in human disease: structure, function and diversity, Trends Microbiol. 3 (1995) 463–468.
[87] J.S. Lee, B.K. Dyas, S.S. Nystrom, C.M. Lind, J.F. Smith, R.G. Ulrich, Immune protection against staphylococcal

enterotoxin-induced toxic shock by vaccination with a Venezuelan equine encephalitis virus replicon, J. Infect. Dis. 185 (2002) 1192–1196.
[88] S. Bavari, M.A. Olson, B. Dyas, R.G. Ulrich, Engineered bacterial superantigen vaccines, Vaccines 96 (1996) 135–141.
[89] C.L. Hatheway, Botulism, in: A. Balows, J.W.J. Hausler, M. Ohashi, A. Turano (Eds.), Laboratory Diagnosis of Infectious Diseases: Principles and Practice, 1988, pp. 111–133.
[90] C.O. Tacket, M.A. Rogawski, Botulism, in: L.L. Simpson (Ed.), Botulinum Neurotoxin and Tetanus Toxin, 1989, pp. 351–378.
[91] L.A. Smith, Development of recombinant vaccines for botulinum neurotoxin, Toxicon 36 (1998) 1539–1548.
[92] J.S. Lee, P. Pushko, M.D. Parker, M.T. Dertzbaugh, L.A. Smith, J.F. Smith, Candidate vaccine against botulinum neurotoxin serotype a derived from a Venezuelan equine encephalitis virus vector system, Infect. Immun. 69 (2001) 5709–5715.
[93] J.S. Lee, A.G. Hadjipanayis, S.L. Welkos, Venezuelan equine encephalitis virus-vectored vaccines protect mice against anthrax spore challenge, Infect. Immun. 71 (2003) 1491–1496.
[94] G.B. Karlsson, P. Liljeström, Live viral vectors: Semliki Forest virus, Methods Mol. Med. 87 (2003) 69–82.
[95] P. Liljeström, H. Garoff, A new generation of animal cell expression vectors based on the Semliki Forest virus replicon, Bio/Technology 9 (1991) 1356–1361.
[96] G. Romano, P. Micheli, C. Pacilio, A. Giordano, Latest developments in gene transfer technology: achievements. perspectives, and controversies over therapeutic applications, Stem Cells 18 (2000) 19–39.
[97] J.A. St. George, Gene therapy progress and prospects: adenoviral vectors, Gene Ther. 10 (2003) 1135–1141.
[98] M.S. Horwitz, Adenoviruses, in: D.M. Knipe, P.M. Howley (Eds.), Fields Virology, vol. 2, 2001, pp. 2301–2326.
[99] R. Vogels, D. Zuijdgeest, R. van Rijnsoever, E. Hartkoorn, I. Damen, M.-P. de Bethune, S. Kostense, G. Penders, M. Helmus, W. Koudstaal, M. Cecchini, A. Wetterwald, M. Sprangers, A. Lemckert, O. Ophorst, B. Koell, M. van Meerendonk, P. Quax, L. Panitti, J. Grimbergen, A. Bout, J. Goudsmit, M. Havenga, Replication-deficient human adenovirus type 35 vectors for gene transfer and vaccination: efficient human cell infection and bypass of preexisting adenovirus immunity, J. Virol. 77 (2003) 8263–8271.
[100] N.J. Sullivan, A. Sanchez, P.E. Rollin, Z.-Y. Yang, G.J. Nabel, Development of a preventive vaccine for Ebola virus infection in primates, Nature 408 (2000) 605–609.
[101] S. Mandl, L. Hix, R. Andino, Preexisting immunity to poliovirus does not impair the efficacy of recombinant poliovirus vaccine vectors, J. Virol. 75 (2001) 622–627.
[102] R. Altmeyer, M. Girard, S. van der Werf, V. Mimic, L. Seigneur, M.-F. Saron, Attenuated Mengo virus: a new vector for live recombinant vaccines, J. Virol. 69 (1995) 3193–3196.
[103] A.A. Khromykh, E.G. Westaway, Subgenomic replicons of the flavivirus kunjin: construction and application, J. Virol. 71 (1997) 1497–1505.
[104] I. Anraku, T.J. Harvey, R. Linedale, J. Gardner, D. Harrich, A. Suhrbier, A.A. Khromykh, Kunjin virus replicon vaccine vectors induce protective Cd8+ T-cell immunity, J. Virol. 76 (2002) 3791–3799.
[105] A. McAllister, A.E. Arbetman, S. Mandl, C. Pena-Rossi, R. Andino, Recombinant yellow fever viruses are effective therapeutic vaccines for the treatment of murine experimental solid and pulmonary metastases, J. Virol. 74 (2000) 9197–9205.
[106] C.A.M. de Haan, L. van Genne, J.N. Stoop, H. Volders, P.J.M. Rottier, Coronaviruses as vectors: position dependence of foreign gene expression, J. Virol. 77 (2003) 11312–11323.
[107] J.P. McGettigan, K. Naper, J. Orenstein, M. Koser, P.M. McKenna, M.J. Schnell, Functional human immunodeficiency virus type 1 (Hiv-1) Gag-Pol or Hiv-1 Gag-Pol and Env expressed from a single rhabdovirus-based vaccine vector genome, J. Virol. 77 (2003) 10889–10899.
[108] N.F. Rose, P.A. Marx, A. Luckay, D.F. Mixon, W.J. Moretto, S.M. Donahoe, D. Montefiori, A. Roberts, L. Buonocore, J.K. Rose, An effective aids vaccine based on live attenuated vesicular stomatitis virus recombinants, Cell 106 (2001) 539–549.
[109] A. Takeda, H. Igarashi, H. Nakamura, M. Kano, A. Iida, T. Hirata, M. Hasegawa, Y. Nagai, T. Matano, Protective efficacy of an aids vaccine, a single DNA priming followed by a single booster with recombinant replication-defective Sendai virus vector, in a macaque aids model, J. Virol. 77 (2003) 9710–9715.
[110] A.A. Haller, M. Mitiku, M. MacPhail, Bovine parainfluenza virus type 3 (Piv3) expressing the respiratory syncytial virus (Rsv) attachment and fusion proteins protects hamsters from challenge with human Piv3 and Rsv, J. Gen. Virol. 84 (2003) 2153–2162.
[111] K. Jooss, N. Chirmule, Immunity to adenovirus and adeno-associated viral vectors: implications for gene therapy, Gene Ther. 10 (2003) 955–963.
[112] T.M. Kündig, C.P. Kalberer, H. Hengartner, R.M. Zinkernagel, Vaccination with two different vaccinia recombinant viruses: long-term inhibition of secondary vaccination, Vaccine 11 (1993) 1154–1158.
[113] J.C. Ramirez, M.M. Gherardi, D. Rodriguez, M. Estaban, Attenuated modified vaccinia virus Ankara can be used as an immunizing agent under conditions of preexisting immunity to the vector, J. Virol. 74 (2000) 7651–7655.
[114] Z.Q. Xiang, G.P. Gao, A. Reyes-Sandoval, Y. Li, J.M. Wilson, H.C.J. Ertl, Oral vaccination of mice with adenoviral vectors is not impaired by preexisting immunity to the vaccine carrier, J. Virol. 77 (2003) 10780–10789.
[115] Z.Q. Xiang, G.P. Gao, A. Reyes-Sandoval, C.J. Cohen, Y. Li, J.M. Bergelson, J.M. Wilson, H.C.J. Ertl, Novel, chimpanzee serotype 68-based adenoviral vaccine carrier for induction of antibodies to a transgene product, J. Virol. 76 (2002) 2667–2675.